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Kinetic studies of alkaline phosphatase extracted from rabbit *(Lepus townsendii)* liver

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Studies were carried out to ascertain some kinetic properties of alkaline phosphatase (ALP) extracted from *Lepus townsendii* liver. Incubation of ALP extract with 4-nitrophenylphosphate (4-NPP) formed the basis for determination of enzyme activity. Spectrophotometric method was used to assay the enzyme activity, and the kinetic constants-maximum enzyme velocity (V_{max}) and Michealis-Menten constant (K_m) were evaluated. The K_m and V_{max} values were 0.5×10^{-3} M and 20×10^{-6} M/min, respectively. Inhibition studies showed that ALP activity was competitively inhibited by 0.67 mM sodium hydrogen orthophosphate (NaH_2PO_4) and the inhibition constant (K_i) was 0.9×10^{-3} M. The optimum pH value for ALP activity was about 9.2, and optimum temperature registered was 45°C. ALP activity exhibited linear Arrhenius relationship at temperature greater than 44.95°C with corresponding catalytic energy of activation (E_a) = 15.23 KJ mole^{-1}. The present study gave insights into characteristic catalytic properties of ALP extracted from *L. townsendii* liver.

Key words: Alkaline phosphatase, *Lepus townsendii,* 4-nitrophenylphosphate (4-NPP), Arrhenius relationship, Michealis-Menten constant.

INTRODUCTION

Orthophosphoric monoester phosphohydrolase (E.C.: 3.1.3.1), also referred to as "alkaline phosphatase" (ALP) is a generic term that describes a group of catalytic proteins sharing the capacity to hydrolyze phosphate esters in alkaline medium (Zhang et al., 2004; Saini et al., 2005). The enzyme contains a zinc atom (Zn^{2+}) near the active site that is viewed to be responsible for its catalytic activity (Le Du et al., 2001; Dean, 2002). Also, activation of ALP activity is facilitated by divalent cations such as magnesium (Mg^{2+}) (Kim and Wyckoff, 1990; Le Du et al., 2001; Dean, 2002) and colbalt (Co^{2+}) (Arise et al., 2008). Conversely, ALPs are inactivated/inhibited by wide range of compounds, depending on the source or/and iso-enzymic form of the enzyme (Hummer and Milla´n, 1991; Hoylaerts et al., 1992). For instance, bone and kidney ALPs are inhibited by urea, whereas those obtained from planceta and gastrointestinal are unaffected but inhibited by phenylalanine (Dean, 2002). Also, ALP extracted from *Escherichia coli* is not inhibited by phenylalanine (Hoylaerts et al., 1992). The wide distribution of ALP in biologic systems coupled with its broad specificity makes the enzyme a readily available parameter for diagnostic and research studies (Garba and Gregory, 2005).

Reports showed that ALP extracts from different tissue/organ and various biological systems exhibit variable but characteristic kinetic properties that serve as basis for distinguishing ALP isoforms (Mayer-Sabellek, 1988; Dean, 2002). Therefore, the aim of the present study is to ascertain some kinetic parameters of ALP in liver extract of *Lepus townsendii.*

MATERIALS AND METHODS

Animal handling/chemicals

Five (5) male rabbits between 8 and 9 weeks old were obtained from the Experimental Animal House, Department of Biological

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Table 1. Properties of alkaline phosphatase extracted from *L. townsendii* liver in various purified steps.

Purification step	Total protein (mg)	Total unit (U)	Specific activity (U/mg)	Purification fold
0.1 M glycine-NaOH buffer	19.80±2.43	9.70±3.01	0.49±2.91	1
4.17 M $(NH_4)_2SO_4$	8.70±1.09	40.28±2.01	4.63±0.91	9.45
Dialysis at 2°C for 24 h	8.00±0.89	40.72±1.12	5.09±0.99	10.39
Sephadex G-100	5.60±1.56	52.81±0.98	9.43±1.09	19.24

The extract specific enzyme activity = 9.43±1.09 unit/mg protein.

Science, Usman Danfodiyo University, Sokoto, Nigeria. The animals were healthy and showed no sign of stress. They were fed *ad libitium* with hay and water for a period of one week for acclimatization before they were been sacrificed. The institutional review board of the Department of Biochemistry, University of Port Harcourt, Port Harcourt, Nigeria, granted approval for this study in accordance with the guidelines of the National Institute for Animal Research, Nigeria. All chemicals used were of analytical grade and were products of Sigma chemical company (St. Louis, Missouri, USA).

Preparation of liver extract of alkaline phosphatase

The extraction and purification of the liver enzyme was by the methods described by Malomo et al. (2003) and Arise et al. (2008), but with minor modifications. The rabbit was dissected and the liver carefully removed and washed in physiological saline solution. Two grammes (2 g) of the liver was homogenized in 40 ml of 0.1 M glycine-NaOH buffer (pH 9.9) solution. The homogenate was centrifuged in a Shermond bench centrifuge at 5000 x g for 40 min. The supernatant constituted the crude enzyme extract and was carefully harvested using a Pasteur pipette. To the supernatant, 4.17 M $(NH_4)_2SO_4$ was gradually added while stirring until 30% saturation was achieved. The solution was stored at 4°C for 1 h to precipitate the enzyme (Hulett et al., 1990). The precipitate was collected by centrifugation at 5,000 x g for 20 min and re-dissolved in 5 ml of 0.1 M glycine-NaOH buffer (pH 9.9) solution. The solubilized enzyme was subjected to dialysis at 2°C for 24 h to remove contaminating salt constituents (Qader et al., 2009), and further purified on a sephadex G-100 column to obtain a highly active ALP fraction. In total, ALP extracts from five (5) rabbits were used. Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as the standard. One unit of ALP activity (U) was expressed as 1 µM of 4-NP produced per min. The procedure for ALP purification is summarized in Table 1. The procedure for ALP purification is summarized in Table 1.

Determination of alkaline phosphatase activity

The enzyme activity represents the rate of hydrolysis of 4-nitro-phenylphosphate to 4-nitrophenol which is described as follows:

$$\text{ALP}$$
$$\text{4-Nitrophenylphosphate (4-NPP)} + H_2O \longrightarrow \text{4-Nitrophenol (4-NP)} + \text{Phosphate ion}$$

The enzyme assay was carried out by method described by Glogowski et al. (2002), but with minor modifications. Five (5) test tubes containing 0.45 ml dilutions of 4, 3.2, 2.4, 1.6 and 0.8 mM of 4-nitrophenylphosphate (4-NPP) solutions were introduced into five (5) corresponding test tubes containing 0.45 ml of 0.1 M glycine-NaOH buffer (pH = 9.9). The 4-NPP/glycine-NaOH buffer mixture

was incubated at 37°C for 30 min. The enzyme reaction was started by adding 0.1 ml of enzyme extract into the various test tubes. At a regular time interval of 5 min for 30 min, 5.0 ml of 0.2 M NaOH was added to the assay mixture to terminate ALP activity. The mixture was transferred into a cuvette and absorbance was measured with a spectrophotometer (SPECTRONIC 20, Labtech – Digital Blood Analyzer®) at maximiun wavelenght (λ_{max}) of 405 nm. The concentrations of 4-NP produced in the various tubes were interpolated from standard curves (not shown).

Inhibition studies using NaH_2PO_4 as inhibitor

The enzyme assay was carried out as described earlier. The glycin-NaOH buffer was substituted with 0.1 M glycine-NaOH buffer/2 mM NaH_2PO_4 mixture (2:1, v/v). Final concentration of inhibitor = 0.67 mM.

Effect of pH on enzyme activity

The activity of alkaline phosphatase was investigated under varying pH values of 8.4, 8.8, 9.2, 9.6, 9.9, 10.0 and 10.4 of 0.1 M glycine-NaOH buffer. A substrate concentration of 4 mM 4-NPP was used for the enzyme assay.

Effect of temperature on enzyme activity

The enzyme assay was carried out by incubating the enzyme mixture containing 4 mM 4-NPP in varying temperature values of 5, 15, 25, 35, 45 and 55°C.

Evaluation of kinetic parameters

The kinetic parameters were evaluated with the plot of the recipro-cal of initial velocities (V_i) against corresponding reciprocal of substrate concentrations [4-NPP], that is, $1/V_i$ versus $1/[S]$ (Lineweaver and Burk, 1934).

Statistical analysis

The experiments were designed in a completely randomized method and data collected were analyzed by the analysis of variance procedure while treatment means were separated by the least significance difference (LSD) incorporated in the statistical analysis system (SAS) package of 9.1 version (2006).

RESULTS
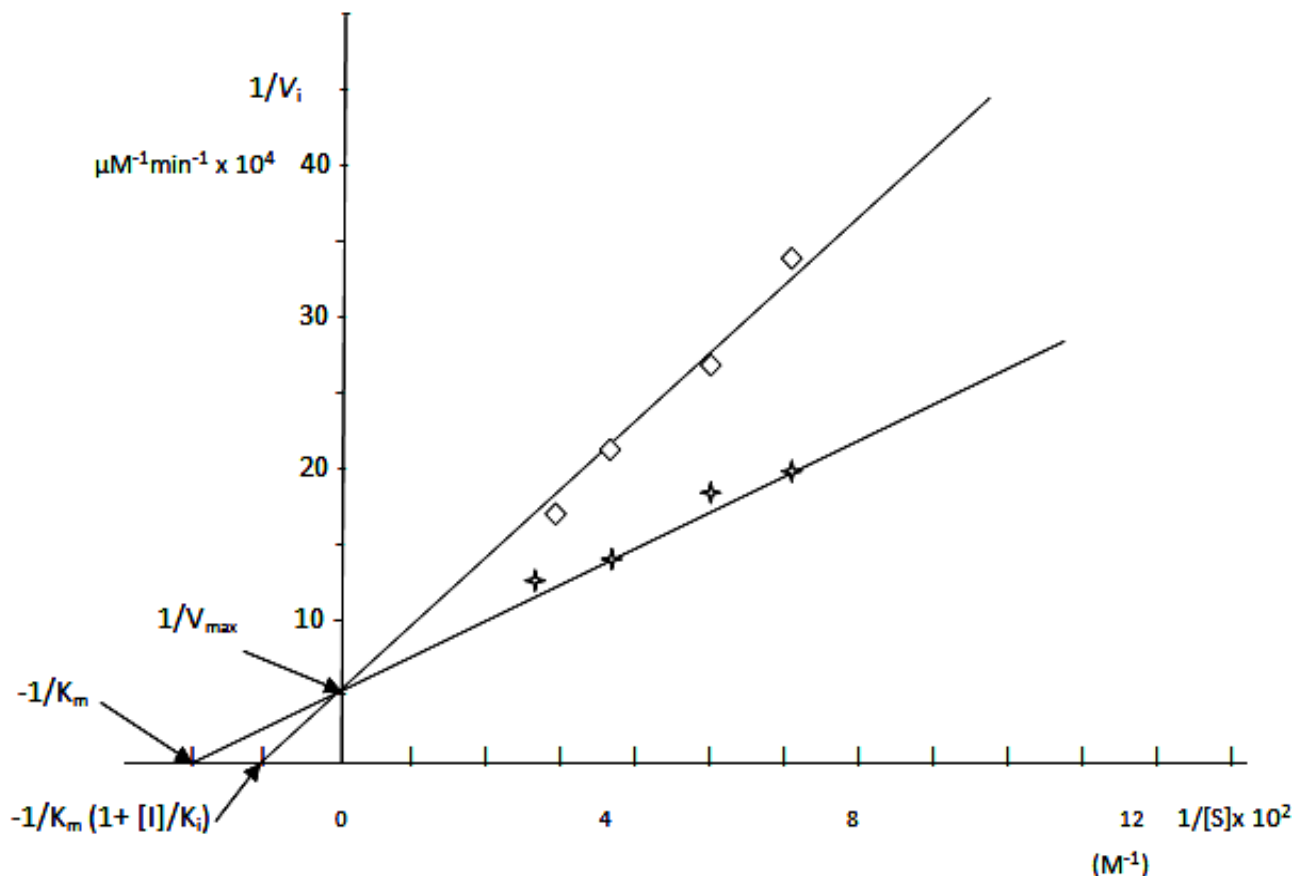
The activity (V_i) of alkaline phosphatase measured in the

Figure 1. Double reciprocal plot of ALP assay in the presence of inhibitor NaH_2PO_4 (   ) and the control (   ) analysis. [I]: concentration of inhibitor = 0.67 mM.

Table 2. Kinetic constants of alkaline phosphatase extracted from *L. townsendii* liver.

K_m (mM)	K_i (mM)	V_{max} (µM/min^{-1})
0.5 ± 0.25	0.9 ± 0.33	20 ± 0.63

Values are Mean ± S.D of five (n = 5) determinations.

absence and presence of inhibitor [(I) = 0.67 mM NaH_2PO_4] is represented by the Lineweaver-Burk plot (Figure 1). By inspection, the Lineweaver-Burk plot (Figure 1) showed that ALP activity was competitively inhibited by 0.67 mM NaH_2PO_4. Also, the plots representing the presence and absence (control) of the inhibitor intersected the x-axis at -1.1 and -2, respectively. Furthermore, the two plots converged at a common point of intersection on the y-axis: 5 x 10^4 µM^{-1}/min^{-1} x 10^4. The kinetic properties of ALP deduced from the Lineweaver-Burk plot are summarized in Table 2. The activity of alkaline phosphatase investigated in varied alkaline pH environment is presented in Table 3.

Values are mean ± S.D of five (n = 5) determinations. Means in the rows with the same letter are not signifi-

cantly different at $p < 0.05$ according to LSD. A cursory look at Table 3 shows that the activity of ALP exhibited two phase profile within the range of values of pH = 8.4 to 10.4. The first phase showed increasing ALP activity as pH values were adjusted from 8.4 to 9.2. Noteworthy, the pH value of about 9.2 corresponded to the maximium enzyme activity attained within the experimental pH range. Therefore, about pH 9.2 represented the optimum pH value of ALP extracted from *L. townsendii* liver. However, alkaline phosphatase activity at pH 10.0 was not significantly different ($p < 0.05$) from the activity at optimum pH. Further increases in pH values beyond the optimum engendered decreasing level of ALP activity.

The relationship between alkaline phosphatase activity and experimental incubation temperature of the enzyme assay is presented in Table 4. The pattern ALP activity in relation to changes in experimental temperature showed two phase profile. The results in Table 4 showed that the optimum temperature of alkaline phosphatase activity was about 45°C. The maximum enzyme activity attained at optimum temperature was 5.01 ± 0.56 µM/min^{-1}. Alkaline phosphatase activity at 35°C (4.67 ± 0.79 µM/min) was not signficantly different ($p < 0.05$) when compared to activity at 55°C (4.53 ± 0.83 µM/min^{-1}). Using

Table 3. Levels of alkaline phosphatase activity with corresponding pH values.

Buffer pH	8.4	8.8	9.2	9.6	10.0	10.4
$[V_i]$ (µM/min^{-1})	4.60 ±0.38[a]	7.23±0.99[b]	11.20±0.76[d]	8.96±0.73[c]	8.80±0.34[c]	7.36±0.66[b]

Values are mean ± S.D of five (n = 5) determinations. Means in the rows with the same letter are not significantly different at p < 0.05 according to LSD.

Table 4. Levels of alkaline phosphatase activity with corresponding temperatures (T °C).

(T°C)	5	15	25	35	45	55
$[V_i]$ (µM/min^{-1}):	2.00±0.46[a]	2.83±0.41[b]	4.00±0.36[c]	4.67±0.79[d]	5.01±0.56[e]	4.53±0.83[c,d]

Values are mean ± S.D of five (n = 5) determinations. Means in the rows with the same letter are not significantly different at p < 0.05 according to LSD.

Arrhenius plot, log V versus $1/T$, the activation energy (E_a) of catalysis was evaluated thus:

Log $V = E_a/2.3RT$

where, R = 8.31447 J K^{-1} mol^{-1}: molar gas constant. But gradient of plot is: -800 = $E_a/2.3R$. Therefore, E_a = 15.23 KJ mole^{-1}.

DISCUSSION

This study demonstrated the activtiy of alkaline phosphatase (ALP) in liver extract of *L. townsendii.* The occurrence and diversity of ALP in biological systems has been reported by several authors (Coburn et al., 1998; Zhang et al., 2004). For instance, Weissig et al. (1993) reported on sequence comparisons between different ALP indicated that about 25 to 30% homology exist between mammalian and *E. coli* alkaline phosphatase. Despite differences in amino acid sequences and three-dimensional structures, all ALPs catalyze the hydrolysis of almost any phosphomonoester with release of inorganic phosphate and alcohol. In addition, although ALP exhibits broad specificity for wide range of phosphorylated substrates, the enzyme displays variable affinity for these substrates depending on the molecular nature of the substrate, isoform and source of the enzyme. Szalewicz et al. (2003) noted that the equivalent enzyme and acid phosphatase of frog (*Rana esculenta*) liver displayed higher affinity for peptidic substrates than for small phosphate esters. Also, Coburn et al. (1998), from their findings stated that in the presence of pyridoxal phosphate as substrate, bovin kidney ALP K_m was 0.42 ± 0.04 µmoll^{-1}, whereas enzyme extracted from the intestine gave 1.6 ± 0.2 µmoll^{-1} and placenta gave 0.42 ± 0.08 µmoll^{-1}. Comparatively, the present investigation showed that ALP liver extract of *L. townsendii* exhibited higher affinity for 4-NPP (K_m = 0.5 ± 0.25 mM) (Table 2) than enzyme extracts from rat (*Rattus novergicus*) kidney (K_m = 6.41 mM) (Arise et al., 2008) and human placental

isoenzyme (K_m = 5.55 mM) (Saini et al., 2005). These findings serve to highlight and confirm the occurrence of isoforms of ALP amongst the mammalian genera.

More than five decades ago, Hilliard et al. (1965), demonstrated that endogenous phosphate interferes with the determination of ALP in urine and suggested that the wide variation in serum inorganic phosphate concentrations in diseases such as uremia or renal tubular disease might interfere with ALP measurements in serum. From inspection of the double reciprocal plot (Figure 1), V_{max} of the enzyme was not altered in the presence of inhibitor. Therefore, the present kinetic studies showed that phosphate salt, NaH$_2$PO$_4$, is a competitive inhibitor of ALP activity. This finding is in concord with previous research reports by Fox (1978), Coburn et al. (1998) and So et al. (2007), in which they noted that ALP is competitively inhibited by physiological concentrations of inorganic phosphate. They further averred that inorganic phosphate is an important physiologic regulator of extracellular ALP activity. Incongrously, the possible effects of endogenous phosphate on phosphatase activity with natural substrates under physiological conditions have not been rigorously investigated (Coburn et al., 1998).

Although the pH of an enzyme often reflects the pH of its normal environment, the optimum value may not be precisely the same in every biological system. Noteworthy, as far back as five decades ago, Garen and Levinthal (1960), reported optimal pH for ALP activity of *E. coli* to be about 8.0, while the bovine enzyme optimum pH was slightly higher (8.5) (Harada et al., 1986). Furthermore, study of ALP from human hydatidiform by Aberomand et al. (2008), gave the optimum pH of 10.8. The current study reported optimum pH value of ALP liver extract of *L. townsendii* to be about 9.2. These findings are consistent with prevoius studies by Zappa et al. (2001), in which they observed that mammalian ALPs possess higher pH optima than procaryotes (*E. coli*) enzyme, and proposed that substitution of two amino acid residues with corresponding histidine molecules in the mammalian enzymes are responsible for this increase in
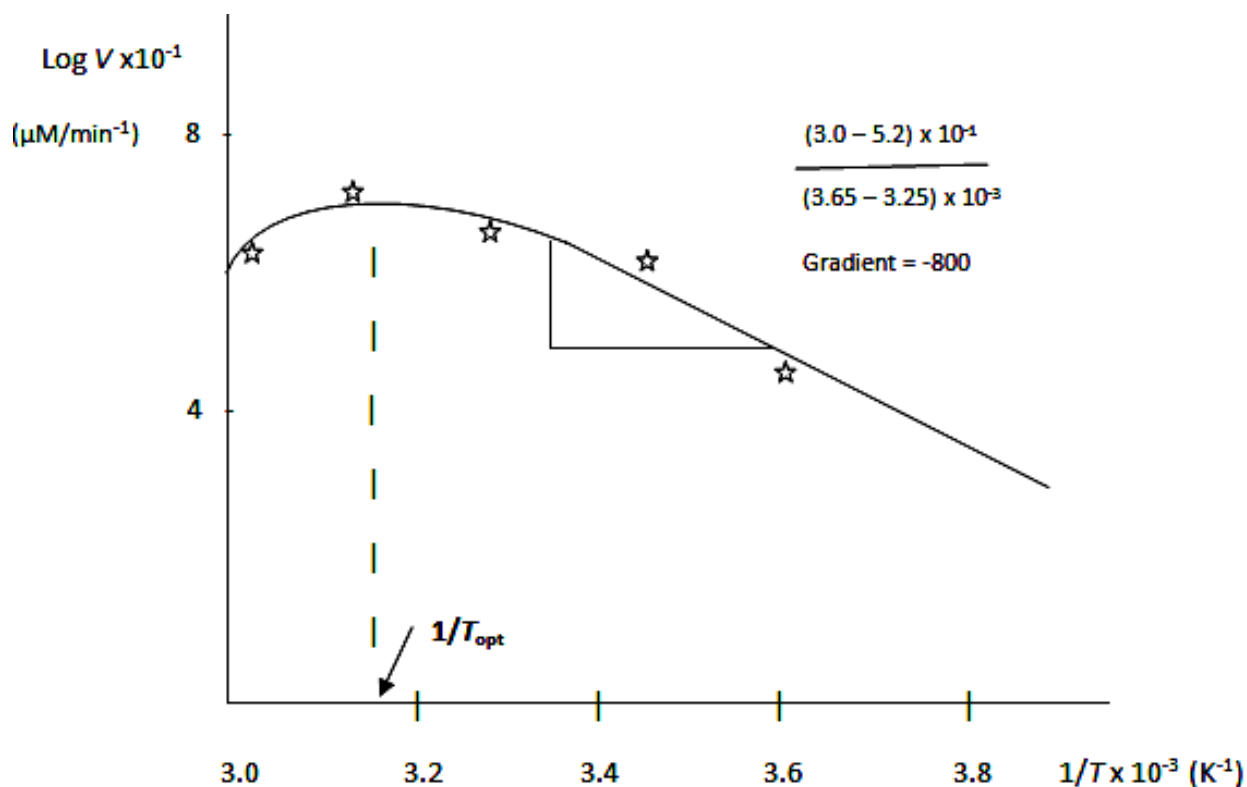
$$\frac{(3.0 - 5.2) \times 10^{-1}}{(3.65 - 3.25) \times 10^{-3}}$$

Gradient = -800

Figure 2. Arrhenius Plot: Effect of temperature on ALT activity. Where $1/T_{opt}$: reciprocal of optimum temperature = 3.144 x 10^{-3} K^{-1}. ALP exhibited linear Arrhenius relationship within the temperature range of: 35 to 55°C.

optimum pH (Holtz and Kantrowitz, 1999; Murphy et al., 1995). These variations in ALP activity which are con-sequences of adjustments in K_m or V_{max} values or both, suggest that pH/activity relationship of ALP may be a factor in the intracellular regulation of its activity.

Enzyme activity/temperature linked properties reported elsewhere (Suzuki et al., 2005; Lee et al. 2007; Copeland et al. 1985; Aberomand et al. 2008) defined the so-called enzyme "temperature optimum" of ALP activity. The present report suggests that at experimental temperature of about 45°C, ALP extract of *L. townsendii* exhibited maximum conformational flexibility to accommondate the substrate and enhanced conformational changes re-quired for maximum catalysis. Contrary to the present suggestion, Daniel et al. (2001) proposed that "tem-perature optimum" is not an intrinsic property, which is derived from a complex mixture of both activity and thermal stability effects.

The Arrhenius plot (Figure 2) conformed to the pattern previousely described by Copeland et al. (1985). They averred that all enzymes of human origin exhibited similar linear Arrhenius relationships with the range of 20 to 37°C with corresponding E_a values between 30 and 36 KJmol^{-1}. In the present study, ALP activity exhibited linear Arrhenius relationship at values of temperature greater than 44.95°C with corresponding E_a = 15.23 KJ mol^{-1} (Figure 2). Although ALP obtained from human and *L.*

townsendii showed linear Arrhenius relation within variable temperature range, E_a values of catalysis were significantly different. Again, in concord with the present investigations, studies by Copeland et al. (1985), reported that porcine kidney enzyme obeyed an Arrhe-nius relationship that was slightly but significantly different from the isoenzymes of human origin.

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